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ON THE TEMPERATURE DEPENDENCE OF INITIAL VELOCITIES OF GLUCOSE TRANSPORT IN THE HUMAN RED BLOOD CELL

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SUMMARY

1. The initial velocity of zero-*trans* efflux of glucose and the equilibrium exchange initial velocity of glucose have been measured in human red blood cells over the temperature range 5–45 °C.

2. Activation energies were calculated and found to be 18.0 ± 0.9 kcal/mole per °K for the zero-*trans* and 16.3 ± 0.9 kcal/mole per °K for the equilibrium exchange process (± S.D.).

3. These findings are shown to be compatible with the tetramer model for sugar transport in erythrocytes.

INTRODUCTION

The rate of a membrane transport process will, in general, depend on the concentration of substrate. One can define a maximum velocity to which the measured velocity tends as the substrate concentration is increased without limit. It is convenient to define two separate experimental situations for determining such maximum velocities. In one type, the zero-*trans* procedure, the substrate concentration at one face of the membrane is kept at limitingly low levels, in theory zero, while the substrate concentration at the other face is varied. The velocity of transport from that face is measured. The maximum velocity for such a procedure is the zero-*trans* maximum velocity, which we will symbolize as V^{zt}. In the second type of experiment the substrate concentration is kept the same at the two faces of the membrane, while the rate of transfer of isotopically labeled substrate is measured across the membrane. This is the equilibrium exchange experiment. The equilibrium exchange maximum velocity is symbolized as V^{ee}. The present paper reports estimates of these two parameters for the glucose transport system of the human red blood cell at a series of temperatures from 5 °C to 45 °C. The resultant data enable us to obtain an estimate of the activation energies of the exchange and zero-*trans* processes of glucose transport over a wide temperature range. The results are considered in terms of theories of membrane transport and are found to be compatible with the predictions of the tetramer model, although they do not in themselves contradict the possibility of carrier-mediated transport.

METHODOLOGICAL CONSIDERATIONS

The maximum velocities of transport can be obtained by extrapolation to infinite substrate concentration of data obtained at finite concentrations. We have, instead, determined the velocities of transport at a single substrate concentration, 300 mM, considered to be sufficient to effectively saturate the transport system. We can do this because at 20 °C the half-saturation concentration for the zero-*trans* efflux of glucose is 25 mM[1], while that for the exchange transport of glucose is 32 mM[2]. At 300 mM glucose concentration, therefore, these two processes are respectively saturated to 92 % and 90 %. At other temperatures in the range studied, the K_m values obtained by Jung *et al.*[3] (exchange transport in red cell ghosts) indicate the percent of saturation of our system to be 93 % at 7 °C, 88 % at 37 °C and 94 % at 22 °C, while from the K_i values of Levine *et al.*[4] (glucose inhibition of sorbose transport) the degree of saturation of our system varies from 95 % at 10 °C to 97 % at 40 °C. Hence, the velocities to be reported in the present study will not differ from the maximum velocity by more than about 10 %. We will therefore refer to our measurements as estimates of the maximum velocities, using the symbols V^{ee} and V^{zt}.

The measure of the initial velocity of the exchange transport can be obtained from the linear plot of the logarithm of the specific activity *vs* time[2]. For the zero-*trans* experiment, a serious technical problem is that of maintaining the external concentration of sugar effectively at zero. Low concentrations of external glucose are sufficient to block the efflux of sugar from the cell[5], a concentration of external glucose of 0.7 mM being sufficient to half-inhibit efflux at 7 °C. This phenomenon complicates enormously the determination of the zero-*trans* efflux rates. To ensure that the transport system is nearly saturated at the inner membrane face, the inside concentration of sugar must be high; to ensure that the true zero-*trans* velocity is measured, the external concentration must be very low. To arrange that these constraints be simultaneously satisfied requires that the zero-*trans* experiments be done in a very high relative volume of medium while the red cell suspension had to be added in high hematocrit. Our observed initial velocities are corrected to take account of the retardation of efflux caused by external glucose (see Results).

EXPERIMENTAL METHODS

A determination of the glucose efflux at a stated time involved (1) the prior loading of the cells with radioactive glucose to 300 mM (120 mM in certain experiments) at 37 °C; (2) vigorous mixing of a sample of cells, brought to the desired temperature, with a large volume of external medium containing 300 mM (or 120 mM) glucose in NaCl–NaH_2PO_4–Na_2HPO_4 buffer, pH 7.4 (saline buffer) or with saline buffer containing an osmotically equivalent quantity of saline, maintained at the desired temperature; followed by (3) the abstraction of samples for subsequent analysis at specified time intervals into an $HgCl_2$–KI–phloretin stopper solution at 0 °C.

The preparation of the cells, loading with glucose, labeling of this glucose, composition of the stopper, and the determination of the radioactivity remaining in the cells as well as the hemoglobin content of the sample were all performed as described in the two earlier papers of the present series[1,2]. The following alterations

were made here for specific purposes: (1) In experiments to test the effect of stirring rate on the efflux of sugar, stirring was accomplished by means of a magnetic stirrer, the speed of rotation of which was calibrated by a Strobolac type 1538-A stroboscope. Stirring rates of 0, 300 and 575 rev./min were chosen. The cells at 50% hematocrit were either swirled gently into 100-ml volumes of external medium, and the stirring then commenced, or else were blown into medium already stirred at the stated rate, with this stirring rate maintained. (2) For experiments at 20 and 25 °C, the room temperature could be controlled to ± 0.5 °C. A water bath controlled to ± 0.1 °C maintained the temperature of the reaction solutions constant before and after mixing. For other temperatures, the same temperature-controlled bath was used, the pre-warmed or -cooled solutions and reaction vessels were taken from the bath onto the magnetic stirrer, the cells added with vigorous mixing, and the reaction vessel returned to the bath within 4 s after mixing. Thereafter, the reaction vessel remained in the bath, since we had shown (Fig. 1) that stirring subsequent to an initial vigorous mixing did not affect the egress of sugar.

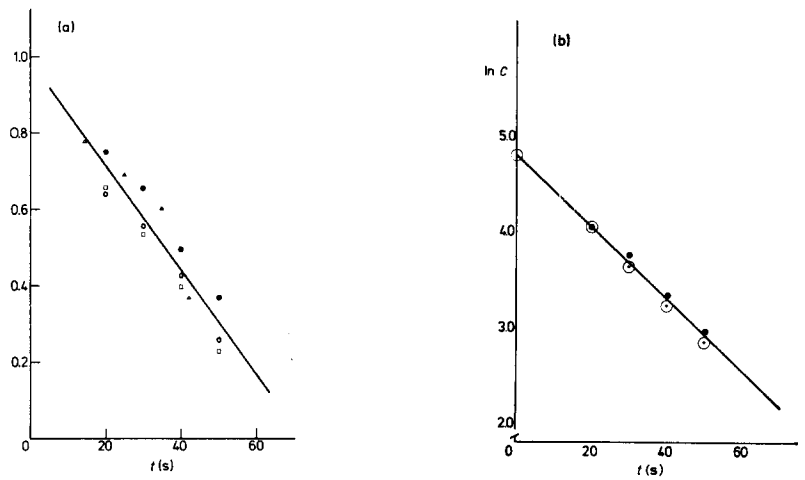

Fig. 1. Effect of stirring on the rate of glucose transport in red blood cells at 20 °C. (a) Fractional activity of labeled glucose remaining in the cells, f, as plotted as a function of time (t) in zero-*trans* experiments. Cells were initially equilibrated with 120 mM glucose. Stirring conditions were as follows: △, cells and zero-*trans* medium were mixed with initial manual swirling of the reaction flask and not stirred thereafter. □, 300 rev./min magnetic stirring upon mixing of erythrocytes and substrate; stirring was then discontinued. ○, 300 rev./min continuous magnetic stirring. ●, 575 rev./min continuous magnetic stirring. The straight line is the equation through all the points (quantities in parentheses indicating standard deviations): $f = -0.82\ (\pm\ 0.04) \times t(s) + 0.99\ (\pm\ 0.02)$. (b) The natural logarithm of the concentration of labeled glucose remaining in the cells in exchange experiments, ln C, plotted against time. Conditions of stirring were: ⊙, 300 rev./min magnetic stirring upon mixing of red blood cells and substrate; no further stirring. ●, 300 rev./min continuous magnetic stirring. The straight line through the points is the equation $\ln C = -2.26\ (\pm\ 0.05) \times t(s) + 4.79\ (\pm\ 0.03)$.

In experiments at 35 °C and above, a heated-stage magnetic stirrer was used to prevent cooling of the sample during the short time that it was outside the bath. (3) To ensure that the concentration of the glucose outside the cells in the zero-*trans* experiments was as low as technically possible, the external volume was made large. In most experiments the ratio of the volume of external medium to cell sample (cells

plus the medium in which they were equilibrated) was 500-fold. In experiments at 15 °C, this ratio was increased to 750-fold; and in the experiments at 5 °C, a 1000-fold volume ratio was used. To further reduce the concentration of external sugar at low temperatures, the cell hematocrit was increased from the usual 50 % to 75 % at 15 °C and 5 °C. Conditions for the zero-*trans* and exchange experiments were identical, as far as volume and hematocrit were concerned, at each temperature.

Each experimental point was performed in quintiplicate (except for those at 45 °C, where a 9-fold repetition was used). We have calculated, for each experimental point of the quintiplicate determinations, the standard deviation from their mean. The average of these standard deviations is 7.4 %. With five determinations of each point, the standard error of an observation is, on the average, 3.3 %. The average standard deviation for the zero-time points, taken separately, is 3.7 %, significantly lower than that from the general mean. Thus a part of the error in our determinations comes from the difficulty of controlling the precise time of the reaction.

At the lowest temperatures, samples could be taken every minute. At the highest temperature, samples were taken after 5 s, and it would have been a decided advantage to take samples at shorter times, had a satisfactory apparatus been available.

RESULTS

We first tested the effectiveness of the stopper solution. Table I shows the results of an experiment in which cells containing labeled glucose at 5 mM and 80 mM were mixed at zero time with a solution containing 30 ml of the $HgCl_2$–KI–phloretin

TABLE I

TEST OF EFFECTIVENESS OF THE MERCURY–PHLORETIN STOPPER SOLUTION[1] IN PREVENTING GLUCOSE LOSS FROM RED BLOOD CELLS AT 0 °C.

Time of contact with stopper solution (min)	*Glucose concentration (mM)* *	*Standard deviation (mM)* *
0	5.00	± 0.24
15	4.98	± 0.09
30	4.51	± 0.14
60	4.25	± 0.10
0	80.0	± 2.1
15	76.4	± 0.8
30	76.0	± 5.2
60	65.1	± 3.2

* Each experiment consisted of triplicate determinations.

stopper solution[1] at 0 °C and 10 ml of saline buffer. This is the concentration of the stopper solution in the conditions of a normal efflux experiment in which 10 ml of the efflux medium containing cells are mixed with 30 ml of the stopper solution at 0 °C. The cells were left in contact with the stopper solution for periods up to 60 min and centrifuged and analyzed in the usual way. Table I shows that in a zero-*trans* experiment at least 90 % of the cellular glucose is retained in the presence of the stopper

solution at 0 °C during the first 30 min of contact. At higher concentrations of glucose such as we used in the present study, the stopper solution will be even more effective, since the loss of a proportionately small amount of glucose will ensure that sufficient glucose is outside the cell to prevent further efflux. In the experiments as later performed, no sample was left in contact with stopper solution for more than 20 min before centrifugation. In a previous publication from this laboratory, a comparable experiment in the effectiveness of the same stopper solution on the exchange transport of glucose was reported[2]; the findings were similar.

We next tested the effect of stirring and the rate of stirring on the efflux of glucose in exchange and net transport[6]. Cells were incubated with radioactive glucose at 120 mM and mixed, as in the legend to Fig. 1, with saline buffer containing 120 mM unlabeled glucose or the osmotically equivalent amount of NaCl. Stirring was discontinued after the initial mixing, or was continued at the fixed rates of 300 or 575 rev./min as indicated. Four different combinations of stirring were tried for the net transport experiments and two in the exchange experiments. In no case was an effect of stirring rate on the egress of glucose observed.

That stirring, after an initial effective mixing, should have no effect on the rate of glucose exit, is not surprising when one considers the relatively large half-time of glucose efflux, some 25 s at 20 °C. A detailed consideration of this point is given in a recent review[7]. It was surprising to read that Naftalin[6] found an apparent effect of stirring on the rate of sugar efflux. His results can only be accounted for, in the face of our inability to reproduce them, on the assumption that in his hands the initial mixing was not sufficiently vigorous. If this were the case, then one might expect that pockets of cells isolated from the external medium might exist in which the concentration of sugar would not conform to that in the exterior. Under these conditions stirring might break up the cell pockets and speed up the efflux of sugar. But this is not a true model for the experiments of other investigators in the field, who have generally ensured that vigorous mixing of cells and medium was achieved at the start of an experiment. Miller[14] has shown by direct cinematographic observation that this procedure yields 90 % complete mixing within 0.7 s.

Having established the effectiveness of our stopping and mixing procedures, we were in a position to study the temperature effect on transport. Fig. 2 shows the time course of efflux in two representative zero-*trans* experiments. The egress values are linear with time within the accuracy of the experiments at all except the highest temperatures. We imposed the reasonable criterion that we would use points in which the glucose concentration in the cells did not fall below 100 mM in the zero-*trans* experiments. This resulted in only three usable points at 45 °C. At 45 °C we solved the problem of the very rapid uptake by doing a 9-fold determination of the 5-s uptake; we equated this value with the initial rate in both the zero-*trans* and exchange cases for mutual comparison. Plots of the logarithm of the counts remaining in the cells *versus* time for two representative exchange experiments are presented in Fig. 3. Table II shows our effective velocities for zero-*trans* and exchange over the temperature range studied.

In our discussion of the zero-*trans* experiment (*cf.* Methodological considerations) we commented on the findings of Sen and Widdas[5] that the accumulation of even low concentrations of glucose retards the rate of efflux, especially at low temperatures. We calculated an approximation of the extent of this retardation from the time-

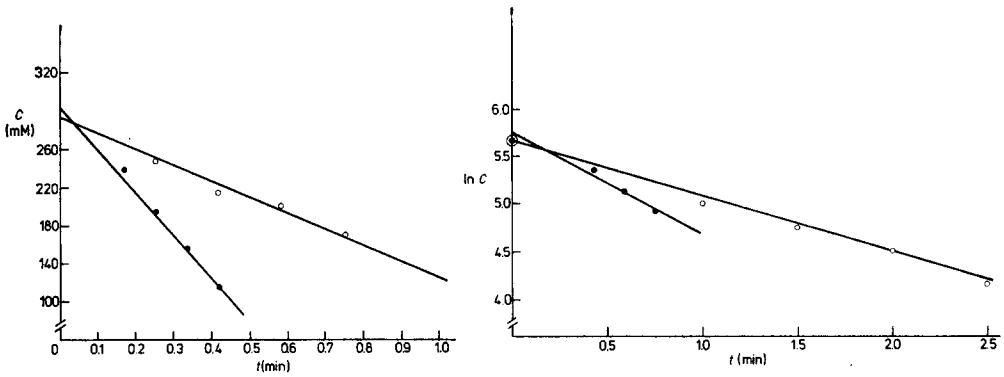

Fig. 2. Glucose efflux from red blood cells in zero-*trans* experiments at 25 and 35 °C. Glucose concentration in the cells, mM (mmoles/l cell water), *vs* time (*t*). ○, 25 °C. The straight line is the equation (standard deviations of the straight line parameters in parentheses) $C = -173\ (\pm\ 11) \times t\ (\text{min}) + 295\ (\pm\ 5)$. ●, A similar experiment at 35 °C. The straight line is given by $C = -448\ (\pm\ 18) \times t\ (\text{min}) + 305\ (\pm\ 5)$.

Fig. 3. Equilibrium exchange of glucose in red blood cells at 15 and 25 °C. The natural logarithm of the activity of cellular glucose (as concentration, *C*, mmoles/l cell water) is plotted against time (*t*). ○, Equilibrium exchange at 15 °C. Linear regression gives the equation $\ln C = -0.606\ (\pm\ 0.020) \times t\ (\text{min}) + 5.673\ (\pm\ 0.034)$, straight line parameters ± standard deviations. ●, Equilibrium exchange at 25 °C. The linear regression line is $\ln C = -1.026\ (\pm\ 0.058) \times t\ (\text{min}) + 5.735\ (\pm\ 0.028)$.

TABLE II

INITIAL VELOCITIES OF GLUCOSE EFFLUX FROM RED CELLS EQUILIBRATED WITH 300 mM GLUCOSE *

Zero-*trans* velocities, V^{1zt}, were determined from plots of the raw data such as Fig. 2. Exchange velocities, V^{ee}, were obtained from treatment of data such as that of Fig. 3. The V^{zt} are estimates of maximum velocities in the zero-*trans* experiments obtained by compensation of the V^{1zt} for retardation of efflux according to Eqn 1 (*cf.* text). The velocities are in units of mmoles/l cell water per min. Each determination is the mean of quintiplicate measurements, except for the 45 °C experiment, where each point is the mean of nine samples.

Temperature (°C)	V^{1zt}	*S.D.*	*No. time intervals taken*	V^{zt}	V^{ee}	*S.D.*	*No. time intervals taken*
5	16	± 4	(10)	23	47	± 2	(5)
15	86	± 14	(5)	103	176	± 6	(5)
20	112	± 10	(5)	133	310	± 7	(10)
25	173	± 11	(5)	206	318	± 18	(5)
35	448	± 18	(10)	492	880	± 62	(5)
40	850	± 34	(3)	970	1360	± 45	(5)
45	1650	± 127	(2)	1760	2390	± 25	(2)

* The exchange experiments at 20 °C were performed with cells loaded to 120 mM. The value of V^{ee} given is the experimental value corrected to the fraction of saturation which would prevail at 300 mM glucose, calculated by the relation

$$V^{ee}_{(300\ \text{mM})} = V^{ee}_{(120\ \text{mM})} \cdot \frac{\frac{300}{300 + K_m}}{\frac{120}{120 + K_m}}$$

where K_m = 32 mM at 20 °C (ref. 2).

averaged external concentration and K_m values for glucose efflux at the appropriate temperatures according to the following equation, which provides a formal description of the phenomenon which Sen and Widdas[5] demonstrated:

$$V^{zt} = V^{1zt} \cdot \frac{K_m + C_{av}}{K_m} \tag{1}$$

where V^{1zt} is the experimental initial rate and C_{av} the mean of the external glucose concentration at the initial instant and the concentration at the time of the last sampling. The extent of retardation of the velocity could vary from 9% at the highest temperature to 29 % at the lowest. Table II shows the velocities as corrected upwards by the appropriate factor, as calculated by Eqn 1. These corrected values of V^{zt} are used in subsequent discussion. (We note that Eqn 1 applies whatever happens to be the molecular basis of the transport event.)

The activation energy of a process which follows Michaelis–Menten kinetics may be determined, in appropriate cases[8], by a plot of log V *vs* $1/T$. Since our systems are very near saturation, and the rates we measure are a good approximation to initial rates, we can determine the activation energy for the two types of processes by plotting log V^{ee} and log V^{zt} *vs* $1/T$. Arrhenius plots of our results are shown in Fig. 4.

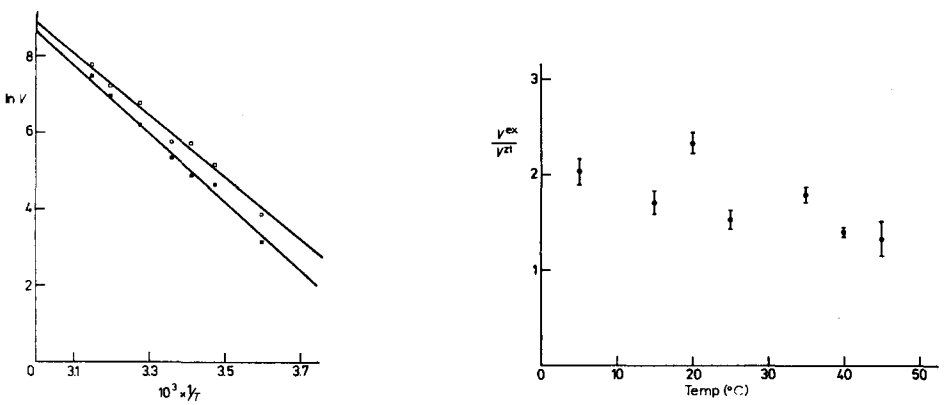

Fig. 4. Activation energies for exchange and for zero-*trans* net flow. ■, zero-*trans*. □, exchange. Linear regression gives the straight lines (± S.D.): $\ln V^{zt} = -9.066\ (\pm\ 0.442) \cdot 10^3 \cdot 1/T + 35.85\ (\pm\ 1.48)$ and $\ln V^{ee} = -8.199\ (\pm\ 0.453) \cdot 10^3 \cdot 1/T + 33.48\ (\pm\ 1.52)$. From the slopes we obtain the activation energies: $E^{ee}_{act} = 16.3 \pm 0.9$ kcal/mole per °K and $E^{zt}_{act} = 18.0 \pm 0.9$ kcal/mole per °K.

Fig. 5. The ratio of the initial velocities of glucose efflux, V^{ee}/V^{zt}, at temperatures in the range 5–45 °C. The data are from Table II, bars indicating one standard deviation.

From the slope of the least squares line we obtain the values (± standard deviation) $E^{zt} = 18.0 \pm 0.9$ kcal/mole per °K and $E^{ee} = 16.3 \pm 0.9$ kcal/mole per °K. We call attention to the close correspondence between the values of the activation energy for the zero-*trans* and exchange processes.

DISCUSSION

There have been a number of previous studies of the variation of kinetic properties of sugar transport with temperature. Lacko and Burger[9] studied the temperature dependence of galactose transport (both exchange and net transport) in the red blood cell. In great contrast to our findings, they report considerably different values, namely, 13.00 kcal/mole per °K for net transport and 4.10 kcal/mole per °K for exchange transport. However, their experiments must be faulted on technical grounds. They did not use initial rates, but rather took 1-min uptakes as a measure of the initial velocity, and it is clear from their Fig. 5 that in many cases the entry of galactose was already 90 % complete at that time. In addition, their experiments are not strictly comparable with ours, because their exchange experiments consisted of measurements of exchange of galactose with glucose, rather than equilibrium exchange of a single sugar. In a recent paper, however, Lacko *et al.*[15], using an improved technique which allows a close approximation of the initial rate to be measured, have found at 0 °C and at 20 °C values of V which are very comparable to our values for exchange, at 5 °C and 20 °C, but which differ considerably from ours for the zero-*trans* experiments. However, it must be pointed out that their measurements are of zero-*trans* influx, whereas ours are for zero-*trans* efflux. Both Sen and Widdas[5], Dawson and Widdas[10] and Bolis *et al.*[11] studied the temperature dependence of net glucose efflux. Both groups reported, and attached importance to their finding, that the Arrhenius plot was nonlinear. In contrast, our Fig. 4 shows a very good linear relationship for the Arrhenius plot of the net fluxes. In the case of the experiment of Sen and Widdas[5], the cells were equilibrated at only 76 mM glucose, which is unlikely to be sufficient to saturate the transport system at the extremes of the temperature range, a factor which could account for their findings. We performed by linear regression an Arrhenius plot of their velocities of efflux, as presented graphically in ref. 5, and have obtained the activation energy 17.4 ± 1.1 kcal/mole per °K. In the experiments of Bolis *et al.*[11] the cells were loaded to 300 mM, so the system was effectively saturated. However, their analyses were performed by the not widely used indirect osmotic method, and are probably not as accurate as the mercury–phloretin stopping method used in our laboratory or the photometric method used by Sen and Widdas[5]. From our experiments we see that at higher temperatures efflux is so rapid that the stopping method used must be essentially instantaneous if large errors are not to be introduced, and we may question whether the indirect osmotic method achieves this. Our calculation of the activation energy of net efflux from the straight line obtained by the least squares method through their data yields the value 13.1 ± 1.5 kcal/mole per °K. Finally, results of the experiments of Jung *et al.*[3] on extensively washed red cell ghosts yield an Arrhenius plot for equilibrium exchange showing perhaps a small deviation from linearity, a phenomenon which we will shortly discuss. Assuming their data to fit a linear Arrhenius plot, we calculated a value of activation energy for exchange of 24.0 ± 3.5 kcal/mole per °K.

Since from our results it appears that the activation energies of the zero-*trans* and exchange transport are not very different, it seems reasonable to suppose that the rate-limiting step is the same in both cases. More insight into the relation between these two processes can be obtained when the data are treated in the following way. For each temperature we calculate the ratio of V^{ee} to V^{zt} and plot it as a function of

temperature (see Fig. 5). It can be seen from the figure that this ratio is fairly constant at about 2 between 5 and 35 °C but appears to drop somewhat thereafter. On the conventional carrier model[12] this ratio is a measure of the rates of movement of the loaded to the unloaded carrier, and there is no known restraint on the value of this ratio. However, on the recently proposed tetramer model[13] this ratio has a more interesting molecular interpretation. The tetramer model assumes that binding of substrate induces a conformation change which allows a transition to occur between two isoenergetic forms of the tetramer. The question arises as to how the rate of the initial conformation change depends on the number of substrate molecules bound, since in an equilibrium exchange experiment at saturation, twice as many substrate molecules will be bound as in a zero-*trans* experiment. When the probability of the substrate-induced conformation change is low, the rate of substrate transport should be proportional to the number of substrate molecules bound. Hence, the ratio of the maximum velocities should be two, as is found in the lower temperatures of Fig. 4. As the temperature rises, the probability of a conformation change increases, and the rate of transport will no longer be directly proportional to the number of substrate molecules bound.

We can develop the argument formally as follows: let P be the probability of the conformation change when substrate is bound from one side of the membrane only. Then $(1-P)$ is the probability that no change occurs. It follows that $(1-P)^2$ is the probability that no change occurs when substrate is bound from both sides of the membrane. Hence $1-(1-P)^2$ is the probability that a change will occur when substrate is bound from both sides of the membrane, *i.e.* the joint probability. Thus when $P = 0.1$, the joint probability is 0.19. Hence the ratio of the maximum velocities will be 1.9, which would be difficult to distinguish from 2. However, when the probability rises to 0.5, the joint probability is 0.75, and the ratio of the maximum velocities will fall to 1.5. These predictions of the tetramer model are borne out by Fig. 5. We note, however, that if the ratio of the maximum velocities could be shown to be substantially below one or above two, the present formulation of the tetramer model would have to be modified. For the glucose transport system, it would seem that rather extreme temperatures would be required to set up such a rejection criterion for the model.

We should note that on these arguments, the conformation change induced by substrate binding is a relatively high probability event (it would appear that $P = 0.5$ in the region between 30 to 40 °C). On the other hand, the presumed transition between the two isoenergetic forms of the tetramer which carries the substrate into and then across the membrane has a high activation energy (18 kcal/mole per °K) and will be an exceedingly low probability occurrence. On this analysis, the Arrhenius plots for equilibrium exchange and zero-*trans* experiments should at low temperatures be two parallel lines with ordinates differing by the natural logarithm of two. As the temperature increases, the plot for the equilibrium exchange should approach and ultimately merge with that for the zero-*trans* experiment. The data of Fig. 4 are consistent with this interpretation, which could be the explanation of the slight curvature found for the Arrhenius plots of the exchange experiments by Jung *et al.*[3]. Thus, our data are quite consistent with the predictions of the tetramer model, and, on this model, provide some further insight into the molecular events occurring during the transport of glucose across the human red cell membrane.

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